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Sex-determining genes on mouse autosomes identified by linkage analysis of C57BL/6J-Y^{POS} sex reversal

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A powerful approach for identifying mammalian primary (gonadal) sex determination genes is the molecular genetic analyses of sex reversal conditions (that is, XX individuals with testicular tissue and XY individuals with ovarian tissue)[1–4]. Here we determined the number and chromosomal location of autosomal and X-linked genes that cause sex reversal in C57BL/6J (B6) mice carrying a Y chromosome of *Mus domesticus poschiavinus* origin (Y^{POS}). B6 XY^{POS} mice develop either as females with exclusively ovarian tissue or as true hermaphrodites with ovarian and testicular tissue. In contrast, the Y^{POS} chromosome is fully masculinizing on most other inbred strain backgrounds. B6-Y^{POS} sex reversal appears to result from the incompatibility of the *Sry* (sex determining region, Y chromosome) allele carried on the Y^{POS} chromosome[5] with B6-derived autosomal or X-linked loci[6–9]. We found strong evidence for the location of one gene, designated *tda1* (testis-determining, autosomal 1), at the distal end of Chromosome (Chr) 4 and a second gene, *tda2*, in the central region of Chr 2. A third gene, *tda3*, on Chr 5 is implicated, but the evidence here is not as strong. We suggest that B6 alleles at these loci predispose XY^{POS} fetuses to ovarian tissue development, but no single locus or combination of loci is necessary and sufficient to cause sex reversal. The TDA proteins may regulate *Sry* expression or form complexes with the SRY protein to regulate other genes, or the *tda* genes may be activated or repressed by the SRY protein.

To map the putative autosomal/X-linked sex determining genes involved in B6-Y^{POS} sex reversal, we conducted a genetic cross using the B6-Y^{POS} and DBA/2J (D2) strains. D2 was chosen because the Y^{POS} chromosome is fully masculinizing in D2 mice and in F1 XY offspring produced from matings between D2 females and B6 XY^{POS} hermaphrodites. We mated (B6 × D2)F1 females to B6 XY^{POS} hermaphrodites and analysed the backcross offspring at 14.5–16 days of development, a time when gonadal development is unambiguous. Of the 250 XY fetuses analysed, 222 developed exclusively testicular tissue and 28 developed ovarian and testicular tissue. Examples of fetal gonads observed are shown in Fig. 1. Recovery of first backcross XY offspring with ovarian tissue was in agreement with our hypothesis that, in the homozygous state, B6-derived alleles at one or more loci can cause sex reversal in XY^{POS} mice. Because a simple model involving a recessive, fully penetrant locus cannot explain the observation of only 28 hermaphrodites out of the 250 XY mice analysed (11%), a more complex model is needed.

We conducted a genome-wide linkage analysis using loci that differed between B6 and D2. Chromosome regions showing a correlation between hermaphroditism and homozygosity for B6 loci are graphically represented in Fig. 2 and presented in Table 1*a*. On the basis of Chi square values alone, a significant deviation from the expected 1:1 ratio of B6-transmitted to D2-transmitted alleles is observed for loci located in the central region of Chr 2, the distal region of Chr 4 and the central region of Chr 5. After correcting for multiple sampling (see Methods), deviations from mendelian expectations remain statistically significant only for the loci on Chr 2 and 4. Because the B6 to D2 ratio for loci typed in the controls is not significantly different from the expected 1:1 ratio, the apparent skewing of loci on Chr 2 and 4 (and 5) among hermaphrodites suggests that loci near the relevant markers influence gonadal development in XY^{POS} mice. We conclude that B6 alleles at genes located in the implicated regions of Chr 2 and 4 play critical roles in B6-Y^{POS} sex reversal. The involvement of a Chr 5 locus is less definitive. In keeping with previous convention, we refer to the Chr 4 locus as *tda1* (testis determining, autosomal-1), the Chr 2 locus as *tda2* and the Chr 5 locus as *tda3*.

Table 1*b* shows genotyping data for only the Chr 2, 4 and 5 markers that gave the highest Chi-square values for the hermaphrodites. Data from the normal males are included. In a backcross, eight equally frequent genotypes are expected for three unlinked genes. For the normal males, all 8 genotypes were present and their distribution did not significantly differ from mendelian expectations. For the hermaphrodites, however, only 4 of the expected 8 genotypes were present.

Closer examination of the data reveals that 27 of the 28 hermaphrodites were homozygous B6 for the marker on Chr 4 and homozygous B6 for either the Chr 2 or the Chr 5 marker. The one exception, hermaphrodite 499, is heterozygous B6/D2 for the distal Chr 4 marker but homozygous B6 for the Chr 2 and Chr 5 markers. As mouse 499 is heterozygous for the entire distal third of Chr 4 (data not shown), we conclude that it did not inherit a Chr 4 containing a recombinational event just proximal to *D4Smh6b*. It is possible that mouse 499 is a phenocopy. For example, Y chromosomal nondisjunction during embryonic development can result in sex chromosomal mosaicism (X0/XYY or X0/XY/XYY) and this can cause hermaphroditism[10,11]. If we assume that ovarian and testicular tissue development in 499 is a phenocopy and remove it from consideration, then ovarian tissue can form in an XY^{POS} mouse if it is homozygous B6 for *tda1* and also homozygous B6 for *tda2* or *tda3*. If we assume that 499 is not a phenocopy, then ovarian tissue can form in an XY^{POS} mouse when it is homozygous B6 for *tda2* and *tda3* even in the absence of homozygosity for the B6 allele at *tda1*. Additional experiments will resolve this question.

A significant proportion of the normal males appear to have the same genotype for *tda1*, *tda2* and *tda3* as the hermaphrodites, yet they failed to develop ovarian tissue (Table 1*b*). This finding is reminiscent of other complex inherited traits. For example, Todd and coworkers found that although there was an association between the presence of certain alleles and development of diabetes in mice from crosses involving the NOD strain, not all mice with the predisposing genotype developed diabetes[12]. Similar results were noted by Rise and colleagues for an inherited epilepsy in mice[13]. Such findings suggest 'an

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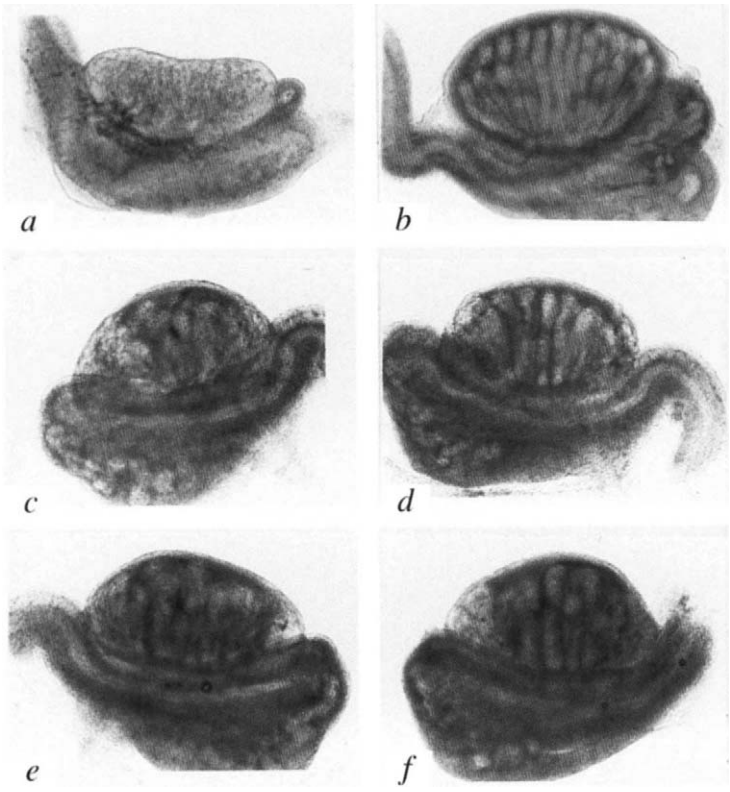

Fig. 1 Freshly dissected gonads with attached mesonephric complexes at 14.5–15 days of fetal development. *a*, *b*, Normal ovarian and testicular development in XX and XY B6 mice, respectively. *c–f*, Ovotestes from XY^{POS} backcross fetuses showing varying degrees of ovarian and testicular differentiation. Ovarian tissue is identified by a reticular appearance and testicular tissue by the presence of cords. In the ovotestes, note ovarian tissue is present at both ends and testicular tissue is located in the centre.

inherited predisposition' to develop a given phenotype or disease. That is, an individual must inherit specific alleles to develop the trait, but this genotype does not, in itself, guarantee development of the trait.

Finally, because 50% of XY mice in the B6-Y^{POS} strain develop as females[6], XY females were expected in this study. No XY females, however, were identified. We suggest that other genes, in addition to the ones identified here, must be in the homozygous B6 state for an XY^{POS} individual to develop exclusively ovarian tissue. If this suggestion is correct, it is not surprising that XY females were not identified in the number of fetuses examined.

We conclude that B6-Y^{POS} sex reversal is a complex trait, as previously predicted[6,7]. An XY^{POS} mouse must inherit a specific set of genes in the B6 homozygous state to develop the abnormality, in this case ovarian tissue. Inheritance of this set of B6 alleles, however, predisposes, but does not in itself, guarantee development of ovarian tissue in XY^{POS} mice. Future investigation may reveal such genes.

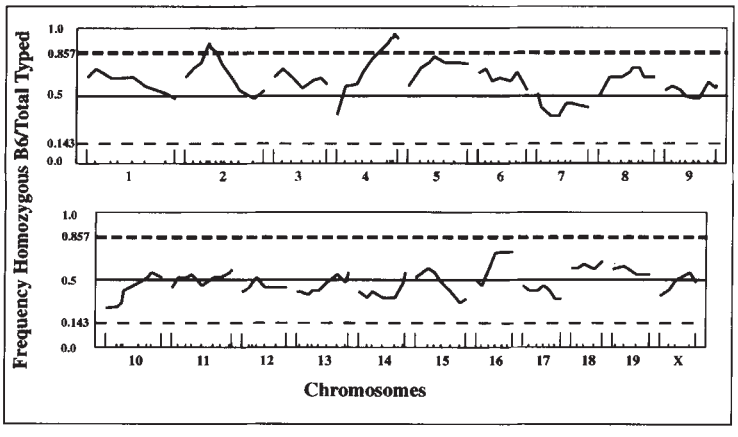

Fig. 2 Scanning the genome for linkage in 28 XY hermaphroditic offspring from the (B6 × D2)F1 × B6-Y^{POS} backcross. Plotted is the frequency of homozygous B6 genotypes among the hermaphrodites at loci spanning Chr 1 through 9 (top tier) and Chr 10 through X (bottom tier). Relative lengths and marker distances along each chromosome are indicated. A B6/B6 genotype frequency of 0.5 (solid line) is expected for chromosomal regions not contributing to sex reversal. A B6/B6 genotype frequency above 0.8957 (thick dashed line) and below 0.143 (thin dashed line) represent statistically significant departures from expected values. Using the criteria of 0.857, *D2Mit88* and *D4Smh6b* are significant at the 5% level, whereas *D5Mit7* is significant at the 7% level. These markers are the ones that gave the highest chi square values for departure from a 1:1 ratio, as indicated in Table 1*a*.

It is noteworthy that no linkage was detected for the distal region of mouse Chr 11, which contains the mouse homologue[14] of the human SRY-type HMG box 9 (*SOX9*) gene[14,15], or for the X chromosome, which contains the mouse equivalent[15] of the human X-linked *DSS* (dosage sensitive sex determination) gene[16]. It is possible that a cross involving B6-Y^{POS} and a strain other than D2 could implicate these chromosomal regions.

There is overwhelming evidence that, on average, 10 cM or larger regions of the human and mouse genomes have remained intact since they last shared a common ancestor[16]. Examination of mouse/human homology maps[17] suggests that the homologue of the mouse Chr 2 *Tda2* gene should be located on human 2q, that the homologue of the mouse Chr 4 *tda1* gene should be located on human 1p, and that the homologue of the mouse Chr 5 *tda3* gene should be on human 4q. So far, no sex reversal phenotypes have mapped to these regions of the human genome. Cloning and characterizing the *tda* genes may provide insight into human sex reversal conditions whose aetiology cannot currently be explained by the presence or absence of a normal *SRY* gene.

By genetic linkage analysis, we demonstrated the existence of two, possibly three, autosomal sex-determining genes whose B6 alleles are functionally incompatible with the *Sry^{POS}*. What are the mechanistic roles of these *tda* genes and their encoded proteins in the pathway of gonadal sex determination? Three things are clearly established about the molecular mechanism of mammalian gonadal sex determination: first, the *Sry* gene encodes the pivotal factor that causes the sexual fate of normal XY embryos to differ from that of normal XX embryos[18–20]; second, the mouse *Sry* gene is expressed transiently during embryonic gonadal development just prior to histologically discernible sex differentiation of the gonad[21]; and third, the Sry protein is a sequence-specific, DNA-binding protein that likely serves to repress and/or activate transcription of other genes[22]. Little is known as to the identity of i) downstream target genes (genes directly activated or repressed by Sry protein), ii) proteins with which Sry protein interacts or iii) factors that regulate *Sry* expression at either the transcriptional or posttranscriptional level. Genetic and molecular studies of the *tda* loci should reveal whether these genes func-

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Table 1 Genotyping data for chromosomes 2, 4 and 5

a, Number of hermaphrodites that are homozygous B6 (B) and heterozygous B6/D2 (B/D) at various marker loci

			Hermaphrodites			Controls[1]	
Chr	Position	Locus	B/B	B/D	Chi-square value[2]	B/B	B/D
2	0	*D2Mit1*	18	10			
	10	*D2Mit6*	20	8		112	101
	19	*D2Mit151*	21	7	7.0		
	26	*D2Mit7*	24	4	14.3	108	105
	28	*D2Mit88*	25	3	17.3		
	30	*D2Mit156*	24	4	14.3	104	109
	37	*D2Mit9*	23	5	11.6		
	43	*D2Mit10*	20	7			
	47	*D2Mit66*	20	8			
	56	*D2Mit17*	17	10			
	65	*D2Mit21*	14	12			
	82	*D2Mit53*	13	14			
	95	*D2Mit200*	15	13			
4	0	*D4Mit149*	10	18			
	10	*D4Mit39*	16	12			
	24	*D4Mit17*	16	11			
	32	*D4Mit9*	17	8			
	43	*D4Mit31*	21	6	8.3		
	62	*D4Mit13*	25	3	17.3	110	103
	66	*D4Mit14*	26	2	20.6	107	106
	68	*D4Mit42*	26	2	20.6	108	105
	69	*D4Smh6b*	27	1	24.1	106	107
	distal end	*Tel4q*	23	2	17.6	67	52
5	3	*D5Mit1*	16	12			
	18	*D5Mit11*	20	8		103	110
	28	*D5Mit15*	21	7	7.0		
	34	*D5Mit7*	22	6	9.1	110	100
	47	*D5Mit25*	21	7	7.0		
	60	*D5Mit65*	21	7	7.0		
	76	*D5Mit99*	20	7			

b, Genotyping information for markers showing the highest chi-square values

	Allele received from the (B6 x DBA)F1 females							
Markers								
D2Mit88	B	D	B	B	D	B	D	D
D4Smh6b	B	B	B	D	D	D	B	D
D5Mit7	B	B	D	B	B	D	D	D
Mice								Total
Hermaphrodites	18	3	6	1	0	0	0	0	28
Males[3]	8	11	9	8	8	12	3	13	72

Chr=chromosome; Position= estimated distance in cM from the centromere to the marker locus using chromosomal positions for MIT markers as given in version 8 from the Whitehead/MIT Center for Genome Research.
[1]Controls consisted of the fetuses comprising the first group (see Methods section).
[2]Only significant chi-square values are given. A full set of data is available on request.
[3]One male was not successfully typed for *D2Mit88*. He was typed as B for two closely linked flanking markers, indicating that he was B for *D2Mit88*.

tion upstream, downstream, or simultaneous with *Sry*. The *tda* genes may be ovarian determinants that are normally activated in XX embryos, repressed in XY embryos but activated in B6 XY^{POS} mice. We had previously postulated the existence of such ovarian determining, *Od*, genes[7]. Of course, the *tda* genes need not all play the same or similar roles in gonadal sex determination.

Methods

Mice. The Y^{POS} chromosome had been backcrossed onto the B6 inbred strain background for more than 40 generations at the time we initiated these experiments. The origin of the Y^{POS} chromosome can be found in refs 6 and 7. To produce backcross fetuses for analysis, (B6 × D2)F1 females were mated to B6-Y^{POS} hermaphrodites. (Some XY^{POS} hermaphrodites develop sufficient testicular tissue to breed as males.) Gonads were removed from fetuses at 14.5–16 d of development and analysed for the presence of ovarian and testicular tissue as previously described[19]. A piece of liver was processed from some fetuses for chromosomal analysis[29] and the remainder of each fetus was frozen for subsequent DNA extraction. In other cases, the entire fetus was frozen for DNA extraction. A total of 217 fetuses (131 females, 73 males and 13 hermaphrodites) were processed and DNA was successfully recovered from all but 2 females and one male. An additional 332 fetuses (168 females, 149 males, and 15 hermaphrodites) were also analysed. For this second group, tissue was kept for DNA extraction only if the fetus was an hermaphrodite. DNA was successfully extracted from all but 3 hermaphrodites. Finally, offspring not analysed during fetal development were examined at three weeks of age after birth. Three offspring with male external genitalia *and* mammary gland-associated yellow pigmented hair (these yellow pigmented hairs are found in B6 XX females, XY^{POS} females and hermaphrodites, but never in B6 XY males) were confirmed by histological analysis to contain ovarian and testicular tissue, and included in the linkage study. In all, 28 hermaphrodites were available for genetic linkage experiments: 13 fetal offspring from the first group, 12 fetal offspring from the second group and 3 weanlings.

Criteria for gonad classification. Gonads with attached mesonephric complex were dissected from individual fetuses and examined using an inverted microscope, as previously described[11]. Analysis at the 14.5–16 d developmental age-range and the use of transmitted light provided optimum conditions for grossly analysing gonadal development. Under these conditions, ovarian tissue is virtually transparent with a distinctive reticular appearance. In contrast, testicular tissue is identified by the presence of seminiferous cords and an incipient tunica albiginea, observed as a contrasting dark rim at the perimeter of the gonad. Using these criteria for ovarian and testicular tissue, small areas of ovarian tissue are easily seen in gonads that have differentiated both ovarian and testicular tissues (ovotestes). See Fig. 1 for examples of ovotestes compared with a normal ovary and testis.

One or both gonads of some fetuses showed undifferentiated tissue at one or both ends of the gonad (13 in the first group and 15 in the second group). This was an expected result, as several *M. domesticus* Y chromosomes (for example, the Y chromosome carried by the AKR/J inbred strain) cause a delay in testicular cord formation at one or both ends of the fetal gonad when present on a B6 inbred strain background[24–26]. In addition, the *M. domesticus poschiavinus* Y chromosome *per se* causes a delay in cord formation but no ovarian tissue development when present on specific genetic backgrounds[23]. If, however, one observes genetically identical fetuses at a later stage of development, the areas that were previously devoid of testicular cords now contain testicular cords. As areas of undifferentiated gonadal tissue can be misclassified as ovarian tissue (that is, lead to the misclassification of a male as a hermaphrodite), and such misclassification has led to some confusion in the literature[9], gonads that were questionable were processed for histological analysis. Gonads that lacked cord formation in the polar regions but contained no ovarian organisation in these cordless regions were classified as testes. By these criteria, it is possible that a very small area of ovarian tissue could be missed. If such mice are present in our data set, their exclusion from the hermaphrodite class would not affect the linkage results but would affect the estimation of the frequency of hermaphroditism in this cross. We can conclusively state that all fetuses classified as hermaphrodites contained both ovarian and testicular tissue.

PCR confirmation of Y chromosomal DNA. To determine the presence of a Y chromosome, a PCR assay was used to detect the multiple copy sequence YB10 that is unique to the mouse Y chromosome[17]. All of the females from the first group of fetuses analysed lacked a Y chromosome. None of the 168 females in the second group were analysed for the presence of a Y chromosome. Given that no XY females were noted in first group, it is reasonable to conclude that none of the females in the second group contained a Y chromosome. All of the males and hermaphrodites in the first group contained a Y chromosome, as did the 12 (out of 15) hermaphrodites in the second group whose DNA was successfully iso-

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Table 2 Probabilities under the hypotheses of no linkage and linkage

k	p (null)	prob (null)	p (alt)	prob (alt)	lod
28	0.5	0.0000000372	1.000	1.000	8.429
27	0.5	0.000000104	0.964	0.374	6.566
26	0.5	0.00000139	0.929	0.277	5.300
25	0.5	0.0000122	0.893	0.237	4.288
24	0.5	0.0000763	0.857	0.211	3.441
23	0.5	0.000366	0.821	0.193	2.723
22	0.5	0.00140	0.786	0.181	2.112

Data are expressed in terms of the number of homozygotes observed out of a total of 28 hermaphrodites typed at a particular locus. k=number of homozygous mice; p (null)=probability of an hermaphrodite being homozygous at a marker under the null hypothesis of no linkage; prob 23(null)=probability of observing k homozygotes under the null hypothesis of no linkage; p (alt)=maximum likelihood estimate of the probability of being homozygous given k; prob (alt)=probability of observing k homozygotes under the alternative hypothesis of linkage; lod=lod score for linkage.

lated. It is reasonable to assume that all males and the 3 hermaphrodites lost in the second group contained a Y chromosome.

Genotyping. Inheritance of a B6- or D2-derived allele from the F1 female parent was determined for all XY hermaphrodites and a subset of the normal males using loci located at 10–20 cM intervals on each autosome and the X chromosome. A total of 241 markers were typed. Genotyping was accomplished by standard Southern blotting methods, for restriction fragment length polymorphism, and PCR amplification, for simple sequence length polymorphism (SSLP) using the method given by the Whitehead/MIT Center for Genome Research[27] or a simplified method[34]. *D4Smh6b* was typed using a single-strand conformational polymorphism assay[34]. When an excess of B6-derived alleles were noted for the hermaphrodites, the remaining fetuses in the first group were typed for this locus to exclude distorted segregation for this chromosomal region.

Linkage data analysis. Linkage between a marker locus and the hermaphroditic phenotype was evaluated through the use of simple tests of association[28]. This involved investigating the statistical significance of a departure from the expected 1:1 ratio of hermaphrodites with homozygous versus heterozygous genotypes at each marker locus. A simple binomial model was used for this purpose. When evidence of departure from the expected 1:1 ratio of homozygotes to heterozygotes was found, the frequency of the homozygous and heterozygous genotypes among control mice (which included all individual mice comprising the first group of fetuses) was obtained to investigate possible segregation distortion of the marker alleles.

The binomial model for assessing the significance of the deviation of the expected 1:1 ratio of homozygotes to heterozygotes was constructed in the following way. Under the null hypothesis of no linkage, the probably (p) that a hermaphrodite possesses a homozygous genotype = 0.5. The probability that k of the 28 hermaphrodites possess a homozygous genotype can be computed from the simple binomial formula:

$$prob(k|p) = \binom{28}{k} p^k (1-p)^{(28-k)}$$

Under the alternative hypothesis that a hermaphrodite-inducing locus is linked to a marker locus, $p>0.5$. The maximum likelihood estimate of p under the alternative hypothesis is simply given as $k/28$. A lod score comparing the hypotheses of linkage and no-linkage for a particular locus can be computed as $\log_{10}$ [prob(k|p = k/28)–prob(k|p = 0.5)]. Table 2 gives relevant probabilities and lod scores for different outcomes. For certain tests involving one degree of freedom in backcross settings, a lod score of 3.3 should be taken as significant, given that an entire genome has been scanned[29]. Thus, from Table 2, only those marker loci showing 24 or more homozygotes offer compelling evidence of linkage. This value can be corroborated by considering the fact that we analysed 152 informative marker loci and invoking Bonferroni-like adjustments for multiple tests. To preserve an α level of 0.05 over all 152 statistical tests performed, we would need to observe a test producing a P value of 0.05/152=0.000329. Only those loci showing 24 or greater homozygotes would produce P values less than this value.

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